



Central kappa opioid receptors modulate salt appetite in rats^{☆,☆☆}

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ABSTRACT

The role of the central opioid system in the control of water and salt intake is complex, with both stimulatory and inhibitory effects having been observed. The aim of the present study was to investigate the participation of the central κ -opioid receptors in the control of salt appetite. Male Wistar rats were submitted to two different experimental protocols: sodium deficit produced by the diuretic, furosemide, and brain angiotensinergic stimulation in rats under normal sodium balance. Lateral ventricle (LV) injections of Nor-binaltorphimine (Nor-BNI) at different doses (5, 10 and 20 nmol) inhibited hypertonic saline solution (1.5%) intake in sodium-depleted rats. The salt appetite induced by an LV injection of angiotensin II (AngII) (10 ng) was also blocked by Nor-BNI injections into the LV, while no significant change was observed in water intake. Furthermore, the decrease in salt intake seems not to have been due to a general inhibition of locomotor activity or to any change in palatability, since central administration of Nor-BNI failed to modify the intake of a 0.1% saccharin solution when the animals were submitted to a “dessert test” or to induce any significant locomotor deficit in the open-field test. Also the central administration of Nor-BNI was unable to modify blood pressure in sodium-depleted animals. The present results suggest that activation of endogenous κ -opioid receptors modulates salt appetite induced by sodium depletion and by central angiotensinergic stimulation in rats.

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1. Introduction

The different families of opioid peptides include endorphins, enkephalins, dynorphins, and nociceptin/orphanin that act through four major types of receptor: mu (μ), kappa (κ), delta (δ) and nociceptin (N/OFQ). There is evidence of distinctive but overlapping distributions of μ , κ , δ and N/OFQ receptors in different areas of the brain [1–7]. The endogenous opioid system is involved in a variety of functions and is found in multiple networks throughout the brain, especially in areas related to feeding and drinking behavior, cardiovascular control and endocrine regulation [1,8]. The role of the brain opioid system in regulating feeding behavior and modulating reward-related responses and the palatability of different substances has been well-documented [8–11]. The participation of the central opioid system in the control of water and salt intake is rather complex, with both stimulatory and inhibitory effects having been observed depending on the anatomical location, the type of opioid receptor involved and the doses of the opioidergic agents used.

The physiological regulation of body sodium depends on a multifaceted mechanism involving homeostatic responses and palatability and reward aspects of salt intake. Sodium deficit motivates behavior to specifically seek out and ingest the sodium ion [12–15]. Some studies have also shown the participation of endogenous opioid peptides in salt preference. The antagonist opioid, naloxone, administered both systemically and in the central nervous system, reduces the intake of hypertonic, hypotonic and isotonic saline solutions which are preferred over water in water-deprived rats [16–19]. Furthermore, in water-deprived rats central administration of μ - and κ -antagonists, but not δ -antagonists, decreases water intake in a two-bottle choice test between water and saline solution (0.6% and 1.7%). However, any of the antagonists used in this study were able to alter the intake of saline solution [20]. Conversely, in non-deprived rats, saline intake is increased by injecting selective δ -, μ - and κ -opioid agonists into the cerebroventricular system and into the parabrachial nuclei [18,19,21]. In addition, systemic injections of morphine increase hypertonic saline intake in sodium-depleted rats [22]. However, in this study, the opioid agents were administered subcutaneously after repeated sodium depletion. It has been shown that repeated sodium depletion may alter the intake of saline solution [23,24], as well as the palatability of salty solutions. Further studies are necessary to clarify the role of the different central opioid peptides receptors in the control of salt appetite.

Because of the contradictions in the literature with respect to the participation of opioid receptors, particularly the κ -opioid receptors, in salt appetite, the objective of the present study was to investigate

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the participation of the central κ -opioid receptors in the control of salt appetite in two different models: 1) sodium deficiency produced by single administration of the diuretic, furosemide; 2) cerebral administration of a natriorexigenic drug, angiotensin II, in rats with normal sodium balance; two approaches that have largely been used in the literature to study salt preference. In addition, the effect of central κ -opioid receptor blockade on locomotor activity, on palatable solution intake (0.1% saccharin) and on blood pressure was investigated. The hypothesis evaluated in this study is that opioid peptides are released during homeostatic challenges and that interaction with κ -opioid receptors may modulate different aspects of salt intake (homeostatic, palatability and reward) in order to correct sodium imbalance.

2. Methods

2.1. Animals

Adult male Wistar rats weighing 220–250 g were used in the present study and kept under controlled light (lights on from 5 AM to 7 PM) and temperature ($22 \pm 2^\circ\text{C}$) conditions. They had free access to tap water and laboratory chow (Nuvital Nutrientes Ltda., Curitiba, Brazil). Groups of rats used in one experimental set were not reused in any other part of the study. All experiments were conducted between 7 AM and 11 AM. The experimental protocols were performed according to the regulations established by the National Council for the Control of Animal Experiments (*Conselho Nacional de Controle de Experimentação Animal* – CONCEA, Brazil).

2.2. Surgical procedures

Five days before the experimental sessions the animals were anesthetized with ketamine/xylazine (80/7 mg/kg i.p.) to enable a guide cannula (22-gauge) to be implanted into the lateral ventricle (LV) according to the following coordinates: anteroposterior = 0.9 mm behind the bregma; lateral = 1.5 mm; vertical = 4.0 mm below the skull. The guide cannula was fixed to the skull with metal screws and dental cement. To avoid obstruction of the guide cannula, an obturator was provided. After surgery, the animals were housed in individual cages and had free access to two different bottles, one containing distilled water and the other containing hypertonic saline solution (1.5%). The animals were handled every day in order to minimize the stress of the experimental procedure. The location of the guide cannula in the LV and the intracerebroventricular injection site was confirmed at the end of the experiment with the use of Evans Blue dye injected through the cannula. The brains were removed, placed in formalin, and later frozen and cut into 40 μm sections. The slices were stained with cresyl violet and analyzed using light microscopy. Only data from the animals in which the tip of the cannula was restricted to the cerebroventricular space and the Evans Blue dye could not be seen in the brain tissue surrounding the ventricle were included in the study.

2.3. Drugs and microinjections

The drugs used were Nor-binaltorphimine (Nor-BNI), an opioid antagonist selective to κ -opioid receptors [25,26], ICI_{199,441} an opioid agonist preferentially binding to κ -opioid receptors [27], both acquired from Tocris Bioscience, Ellisville, MO, USA, and angiotensin II (AngII), which was purchased from Sigma Chemical, Co., St. Louis, MO, USA. The doses of the drugs used in this study were compatible with those used by other research groups: the doses of Nor-BNI were 5, 10 and 20 nmol/rat [20], while the dose of ICI_{199,441} was 9.4 nmol/rat [28] and the dose of AngII was 10 ng/rat [29]. pH was neutral in all solutions and no acid or basic solutions were injected. Central injections were given using a Hamilton microsyringe connected to a 30-gauge injector through polyethylene tubing (PE10).

A total volume of 2 μl was slowly injected (60 s). Furosemide, a loop diuretic, was purchased from Sanofi-Aventis Ltd., São Paulo, Brazil.

2.4. Sodium depletion (experiments 1 and 2)

Animals received a subcutaneous injection of furosemide (10 mg/kg) to induce renal sodium loss 24 h prior to the experimental sessions. After the injections, the rats had free access to distilled water and the standard rat chow was replaced by a low sodium diet (0.001% Na⁺ and 0.33% K⁺). Control animals not submitted to sodium depletion received subcutaneous injections of isotonic saline solution instead of furosemide. In *experiment 1*, the participation of central kappa opioid receptors in the salt appetite of sodium-depleted rats was tested in different groups of sodium-depleted animals receiving LV injections of Nor-BNI at different doses (5, 10 and 20 nmol). In *experiment 2*, another group of sodium-depleted animals received an LV injection of 20 nmol of Nor-BNI plus ICI_{199,441}, a κ -opioid receptor agonist, at a dose of 9.4 nmol, to confirm the specificity of Nor-BNI. Sodium-depleted control animals received LV injections of isotonic saline solution. Bottles containing hypertonic saline solution (1.5%) and distilled water were reintroduced into the cages 15 min after the injections. The first measurement of fluid intake was recorded 5 min after this and measurements continued for the next 120 min. In an additional control group, the animals received subcutaneous injections of isotonic saline solution instead of furosemide and LV injections of isotonic saline solution.

2.5. Central angiotensinergic stimulation (experiment 3)

To study the participation of κ -opioid receptors in water and salt intake under conditions of normal sodium balance, pharmacological stimulation of central angiotensinergic pathways was performed. Different groups of rats received LV injections of Nor-BNI at different doses (5, 10 and 20 nmol) 15 min before receiving AngII (10 ng/rat). Bottles containing 1.5% saline solution and distilled water were made available immediately after the LV injections of AngII. As in the previous experimental sets, the first measurement of fluid intake was recorded 5 min afterwards and measurement continued for the next 120 min. In an additional control group, the animals received central administration of saline instead of AngII.

2.6. Open field test (experiment 4A)

Different groups of rats receiving LV injections of Nor-BNI (20 nmol) or saline solution were submitted to an open field test to exclude the possibility that this agent could have induced a locomotor alteration that would explain the inhibition of salt intake observed here. In this test, the animals were placed in a circular acrylic box (60 cm in diameter and 60 cm high) with an open top 30 min after an injection of Nor-BNI or saline solution into the LV. The floor was divided into eight areas of equal size with a circle at the center (42.43 cm). Hand operated counters and stopwatches were used to score locomotion over a 10 min period by measuring the number of areas into which the rats entered with all four paws. The behavioral experiments took place in a sound-attenuated, temperature-controlled ($24 \pm 1^\circ\text{C}$) room between 7 AM and 11 AM. A white-noise generator provided constant background noise and the apparatus was cleaned with 70% ethanol and dried before each session to minimize olfactory cues.

2.7. Dessert test (experiment 4B)

The effect of an LV injection of Nor-BNI on the intake of a 0.1% saccharin solution, a well-established model of hedonic behavior in rats [30], was used to exclude the possibility of a non-specific, general inhibition of the central nervous system induced by this agent. In this experiment, after LV cannulation the animals were kept in the usual individual cages during the training period with free access only to

tap water and were transferred to a different cage (the test cage) for 2 h each day for seven consecutive days. In the test cage the animals had access to two bottles containing water and 0.1% saccharin solution. After this training period, the animals were divided into two groups and were deprived of fluid for 24 h. Afterwards, the animals received LV injections of Nor-BNI (20 nmol) or saline (controls) 15 min before being transferred to the test cage, and water and saccharin intake was recorded over a 120 min period.

2.8. Blood pressure recording (experiment 5)

One day before the experimental sessions, a catheter (PE50) filled with heparin solution (1000 U/ml) was inserted into the left carotid artery under ketamine/xylazine anesthesia, and exteriorized at the nape of the animal's neck to permit blood pressure recording. At the experimental session, the carotid catheter was connected to a pressure transducer whose signal was amplified and digitally recorded by an analog-to-digital interface (AqDados, version 5, Lynx Tecnologia Eletrônica LTDA, São Paulo, Brazil) and recorded (1 kHz) on a microcomputer for posterior analysis. Distinct groups of sodium-depleted animals received an LV injection of Nor-BNI (20 nmol) or saline solution (controls) 10 min after baseline blood pressure was recorded. In each of these groups, blood pressure continued to be recorded for the next 120 min after Nor-BNI or saline solution was injected. The experimental protocol used in this case was identical to that used to study salt intake in the previous groups. Mean arterial pressure (MAP) was calculated from systolic and diastolic pressure measurements using the AcqKnowledge software program, version 3.5.7, developed by Biopac Systems, Inc., California, USA. The MAP values at the end of the stabilization period (time zero) were used as references to calculate the delta values that are presented throughout the experiments.

2.9. Statistical analysis

The data are presented as means \pm SEM. Statistical analyses were performed using the GraphPad Prism software (GPAD, version 5.0, San Diego, USA). To compare the effect of each treatment on sodium appetite, the cumulative water and sodium intake were analyzed using one-way ANOVA followed by the Student–Newman–Keul's *post-hoc* test at each measurement interval. Student's *t*-test was used to analyze the data obtained in the open field and dessert tests. The delta values of MAP were analyzed using two-way repeated measures ANOVA followed by the *post-hoc* Bonferroni test. Differences between the groups were considered statistically significant when $p < 0.05$.

3. Results

3.1. Experiment 1 – Central effect of a kappa opioid receptor antagonist on salt appetite in sodium-depleted rats

Fig. 1 (panel A) shows the effect of LV injections of Nor-BNI at the doses of 5 ($n = 6$), 10 ($n = 14$) and 20 nmol ($n = 14$) on cumulative salt intake in sodium-depleted rats. As expected, the intake of hypertonic saline was significantly lower in each measurement period in the normonatremic animals receiving LV injections of isotonic saline ($n = 9$) when compared to sodium-depleted rats also receiving LV injections of isotonic saline ($n = 10$). The blockade of central κ -opioid receptors by LV injection of the antagonist Nor-BNI induced a dose-dependent inhibition of salt intake in sodium-depleted rats. At the highest dose used (20 nmol), Nor-BNI significantly inhibited salt intake in the sodium-depleted rats when compared to the sodium-depleted control group. This inhibition of salt intake was evident in all the measurement periods, beginning at the 0–15 min measurement period. At the intermediate dose of Nor-BNI (10 nmol), salt

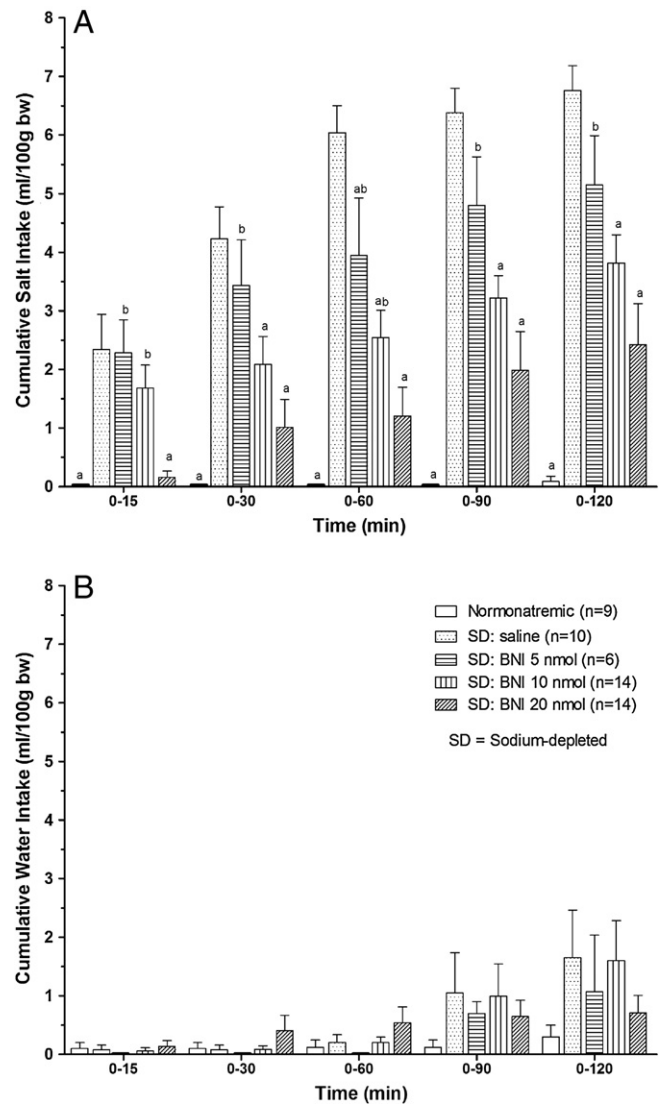


Fig. 1. Cumulative salt (panel A) and water intake (panel B) following lateral ventricle injections of Nor-BNI at different doses (5, 10 and 20 nmol) or saline in sodium-depleted (SD) rats. An additional group of animals not submitted to sodium depletion, receiving lateral ventricle injections of saline, is also shown (normonatremic). The pharmacological treatment and the number of animals used in each group are shown in panel B. Data are presented as means \pm SEM (error bar above). The letter "a" indicates a statistically significant difference (one-way ANOVA followed by Student–Newman–Keul's test; $p < 0.05$) when the different groups are compared to sodium-depleted animals receiving saline at each measurement period. The letter "b" indicates a statistically significant difference when the different groups are compared to sodium-depleted rats receiving 20 nmol of Nor-BNI at each measurement period. Each bar in the graph was obtained from a naïve group of animals.

intake was significantly inhibited compared to that of the sodium-depleted animals treated with isotonic saline. In this case, inhibition started at the 0–30 min measurement period and persisted until the end of the experiment. The salt intake in sodium-depleted rats receiving an LV injection of Nor-BNI at the lowest dose (5 nmol) was similar to that found in the group of sodium-depleted rats receiving LV injections of isotonic saline. Furthermore, the salt intake in sodium-depleted rats receiving 5 nmol of Nor-BNI was significantly different throughout the entire experimental period compared to that of the animals treated with Nor-BNI at the dose of 20 nmol. The one-way ANOVA results are: 15 min – $F_{(4,48)} = 8.617$, $p < 0.0001$; 30 min – $F_{(4,48)} = 10.50$, $p < 0.0001$; 60 min $F_{(4,48)} = 19.99$, $p < 0.0001$; 90 min $F_{(4,48)} = 19.35$, $p < 0.0001$; 120 min $F_{(4,48)} = 17.34$, $p < 0.0001$.

Fig. 1 (panel B) shows the effect of LV injections of Nor-BNI at the doses of 5, 10 and 20 nmol on water intake in sodium-depleted rats.

As expected, water intake was negligible in sodium-depleted rats and remained unaltered by any of the treatments. The one-way ANOVA results are: 15 min – $F_{(4,48)} = 0.301$, $p = 0.876$; 30 min – $F_{(4,48)} = 1.009$, $p = 0.412$; 60 min – $F_{(4,48)} = 1.129$, $p = 0.354$; 90 min – $F_{(4,48)} = 0.881$, $p = 0.482$; 120 min – $F_{(4,48)} = 0.905$, $p = 0.469$.

3.2. Experiment 2 – Central effect of the kappa opioid receptor agonist, ICI_{199,441}, on the inhibitory action of Nor-BNI on salt appetite in sodium-depleted animals

Fig. 2 (panel A) illustrates the effect of ICI_{199,441} (9.4 nmol, $n = 13$) on the inhibition of salt intake induced by Nor-BNI (20 nmol, $n = 11$) in sodium-depleted rats. As expected, saline-treated normonatremic animals ($n = 9$) drank significantly less hypertonic saline compared to sodium-depleted rats also receiving LV injections of isotonic saline ($n = 10$). As in the previous experiment, at the dose of 20 nmol, Nor-BNI exerted a significant inhibitory effect on salt intake throughout the entire measurement period compared to sodium-depleted rats receiving isotonic saline injections. Cumulative salt intake was significantly higher at all measurement periods in sodium-depleted rats receiving 9.4 nmol of ICI_{199,441} but pretreated with 20 nmol of Nor-BNI ($n = 15$) compared to the group treated with Nor-BNI alone. Indeed the amount of salt intake in this group (Nor-BNI + ICI_{199,441}) was similar to that observed in saline-treated, sodium-depleted rats in each measurement period. Central administration of ICI_{199,441} alone failed to alter salt intake when compared to saline-treated, sodium-depleted rats. The one-way ANOVA results are: 15 min – $F_{(4,53)} = 8.611$, $p < 0.0001$; 30 min – $F_{(4,53)} = 15.75$, $p < 0.0001$; 60 min – $F_{(4,53)} = 27.26$, $p < 0.0001$; 90 min – $F_{(4,53)} = 23.24$, $p < 0.0001$; 120 min – $F_{(4,53)} = 26.14$, $p < 0.0001$.

Fig. 2 (panel B) shows the effect of LV injections of the κ -opioid antagonist (Nor-BNI 20 nmol) plus the agonist (ICI_{199,441} 9.4 nmol) on water intake in sodium-depleted rats. As expected, water intake was very low in sodium-depleted rats and remained unaltered by any of the treatments. The one-way ANOVA results are: 15 min – $F_{(4,53)} = 1.478$, $p = 0.222$; 30 min – $F_{(4,53)} = 1.462$, $p = 0.227$; 60 min – $F_{(4,53)} = 1.006$, $p = 0.413$; 90 min – $F_{(4,53)} = 1.176$, $p = 0.332$; 120 min – $F_{(4,53)} = 0.709$, $p = 0.589$.

3.3. Experiment 3 – Central effect of kappa opioid receptor antagonist on salt appetite induced by angiotensin II

Fig. 3 (panel A) shows the effect of LV injections of Nor-BNI at the doses of 5 ($n = 9$), 10 ($n = 14$) and 20 nmol ($n = 10$) on cumulative salt intake induced by central angiotensinergic stimulation in rats. The blockade of central κ -opioid receptors impairs the salt intake induced by stimulation of the central angiotensinergic system in a dose-dependent way. Predictably, salt intake was very low in animals receiving an LV injection of isotonic saline ($n = 9$) throughout the entire duration of the experiment (0.3 ± 0.2 ml/100 g b.w.), while salt intake was high in animals treated with 10 ng of AngII ($n = 10$), beginning at the 0–15 min measurement period and lasting throughout the 0–120 min period (5.4 ± 0.27 ml/100 g b.w.). The salt intake induced by AngII was significantly inhibited by pretreatment with Nor-BNI at the dose of 20 nmol beginning at the 0–30 min period and lasting throughout the 0–120 min period (1.1 ± 0.6 ml/100 g b.w.). At the intermediate dose, Nor-BNI (10 nmol) pretreatment also inhibited salt intake induced by AngII and the amount of salt intake at the 0–120 min period was 2.6 ± 0.66 ml/100 g b.w. The salt intake in animals pretreated with the lowest dose of Nor-BNI (5 nmol) and receiving AngII was similar to that of the group pretreated with saline (saline + AngII). Furthermore, in this group, salt intake was significantly different from that found in the animals pretreated with Nor-BNI at the dose of 20 nmol at the 0–90 min measurement period and this difference persisted until the end of the experiment (3.9 ± 0.55 ml/100 g b.w.). The one-way ANOVA results are: 15 min – $F_{(4,47)} = 3.892$, $p = 0.008$; 30 min –

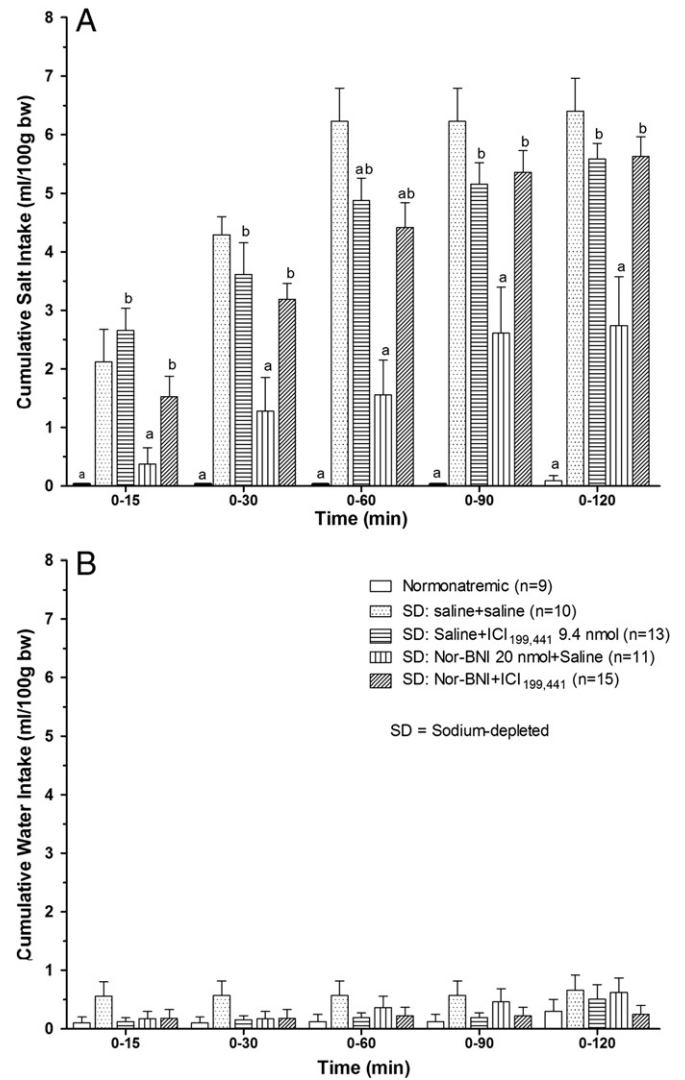


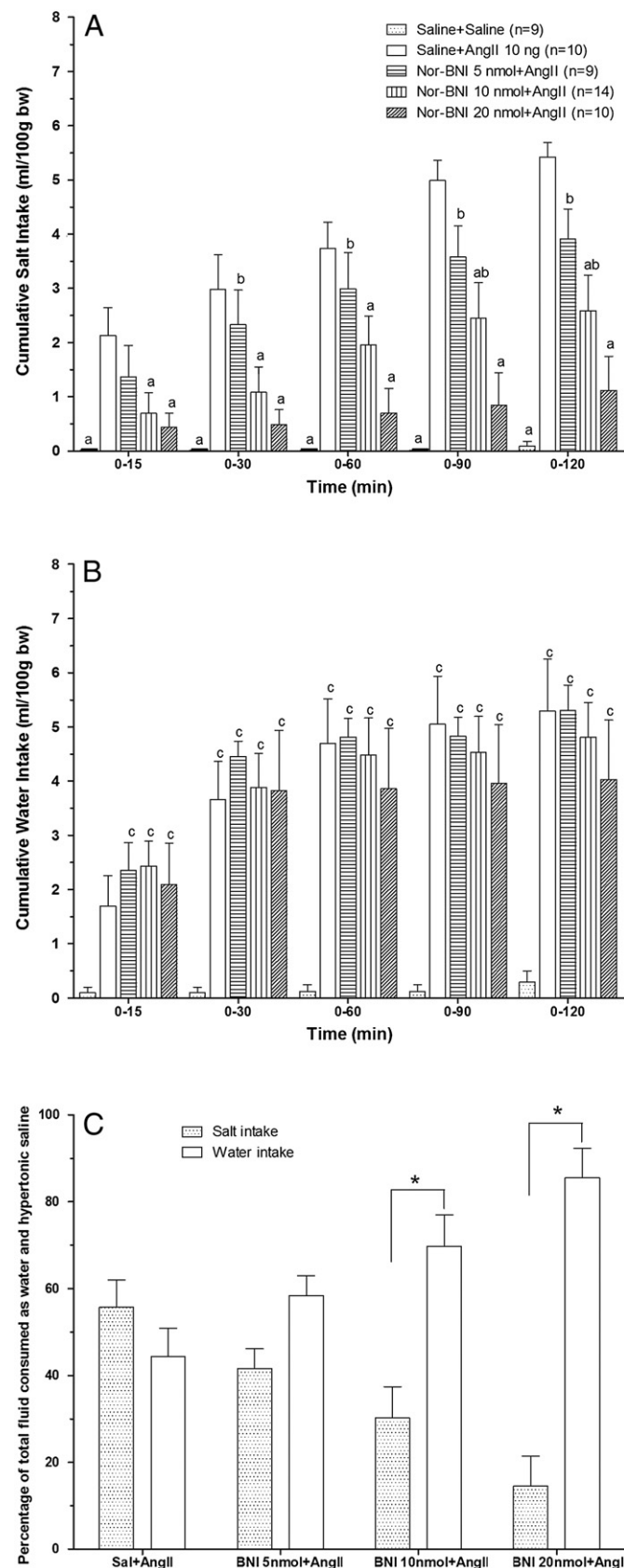
Fig. 2. Cumulative salt (panel A) and water intake (panel B) in sodium-depleted animals pretreated with lateral ventricle injections of ICI_{199,441} (9.4 nmol) or saline before receiving central administration of Nor-BNI (20 nmol), and in control animals receiving two subsequent lateral ventricle injections of saline. An additional group of animals not submitted to sodium-depletion, receiving two subsequent lateral ventricle injections of saline, is also shown (normonatremic). The pharmacological treatment and the number of animals used in each group are shown in panel B. Data are presented as means \pm SEM (error bar above). The letter “a” indicates a statistically significant difference (one-way ANOVA followed by Student–Newman–Keul’s test; $p < 0.05$) when the different groups are compared to sodium-depleted animals receiving saline + saline at each measurement period. The letter “b” indicates a statistically significant difference when the different groups are compared to sodium-depleted rats receiving 20 nmol of Nor-BNI + saline at each measurement period. Each bar in the graph was obtained from a naïve group of animals.

$F_{(4,47)} = 6.305$, $p = 0.0004$; 60 min – $F_{(4,47)} = 8.865$, $p < 0.0001$; 90 min – $F_{(4,47)} = 12.53$, $p < 0.0001$; 120 min – $F_{(4,47)} = 14.27$, $p < 0.0001$.

Fig. 3 (panel B) shows the effect of LV injections of Nor-BNI at the doses of 5, 10 and 20 nmol on water intake induced by central angiotensinergic stimulation in rats. As expected, in rats receiving a LV injection of isotonic saline, water intake was very low throughout the entire duration of the experiment (0.3 ± 0.2 ml/100 g b.w.), while water intake was higher in animals treated with 10 ng of AngII beginning at the 0–15 min measurement period and lasting until 120 min (5.3 ± 0.9 ml/100 g b.w.). Nor-BNI failed to alter water intake induced by LV injection of AngII (10 ng) at any of the doses used (5, 10 or 20 nmol) throughout the entire period of the experiment. The one-way ANOVA results are: 15 min – $F_{(4,47)} = 2.923$, $p = 0.031$; 30 min – $F_{(4,47)} = 5.656$, $p = 0.0008$; 60 min – $F_{(4,47)} = 6.327$, $p = 0.0004$;

90 min — $F(4,47) = 6.808$, $p = 0.0002$; 120 min — $F(4,47) = 6.794$, $p = 0.0002$.

Fig. 3 (panel C) shows the percentage of total fluid intake as water and hypertonic saline at the end of the experimental session (120 min)



after reintroduction of the bottles into the cages). One-way ANOVA values are $F(7,72) = 10.46$, $p < 0.0001$. Unlike the previous experimental sets, during this experimental session, central angiotensinergic stimulation induces both water and salt intake. In the group pretreated with Nor-BNI at the dose of 5 nmol the amount of salt intake was similar to the amount of water intake. However, at the doses of 10 and 20 nmol, salt intake was significantly lower (30.2% and 14.5% respectively) than water intake (69.8% and 85.5% respectively).

3.4. Experiment 4 – Central effect of the kappa opioid receptor antagonist on locomotor activity and hedonic behavior in rats

Fig. 4 (panel A) shows the results of the open field test. Central blockade of κ -opioid receptors failed to alter locomotor activity in sodium-depleted rats. Locomotor behavior was similar in the group treated with an LV injection of Nor-BNI at the dose of 20 nmol ($n = 10$) and in the group of sodium-depleted animals receiving LV injections of saline solution ($n = 11$).

Fig. 4 (panel B) shows the results of the dessert test. The hedonic behavior represented by preference for a palatable solution was confirmed by the higher saccharin intake compared to water intake in saline-treated control animals. At the dose of 20 nmol, Nor-BNI ($n = 6$) injected into the LV failed to alter this hedonic preference. Indeed, animals receiving this κ -opioid antagonist drank similar amounts of saccharin (6.8 ± 0.5 ml/100 g b.w.) and water (3.4 ± 1.0 ml/100 g b.w.) as saline-treated controls (saccharin = 7.5 ± 0.7 ml/100 g b.w. and water = 3.4 ± 1.2 ml/100 g b.w.; $n = 7$).

3.5. Experiment 5 – Central effect of the kappa opioid receptor antagonist on blood pressure in sodium-depleted rats

Fig. 5 shows the effects of Nor-BNI (20 nmol, $n = 8$) on blood pressure in rats submitted to sodium depletion using the same experimental protocol used to study salt intake. In this situation, central administration of Nor-BNI failed to modify blood pressure in sodium-depleted animals compared to sodium-depleted controls (saline-treated, $n = 6$) throughout the entire experimental period.

4. Discussion

In the present study, the data obtained clearly show that: 1) pharmacological blockade of central κ -opioid receptors by a selective antagonist, Nor-BNI, results in a significant decrease in salt intake in sodium-depleted rats; 2) the blockade of those receptors also inhibits the salt intake induced by central angiotensinergic stimulation; 3) the inhibitory effect on salt intake in sodium-depleted rats induced by the blockade of central κ -opioid receptors is impaired by central administration of a κ -opioid receptors agonist; 4) the dessert test revealed that the hedonic ingestive behavior of a palatable saccharin solution

Fig. 3. Cumulative salt (panel A) and water intake (panel B) in animals pretreated with lateral ventricle injections of Nor-BNI at different doses (5, 10 and 20 nmol) or saline prior to receiving central administration of 10 ng of angiotensin II (Ang II) and in control animals receiving two subsequent lateral ventricle injections of saline. The pharmacological treatment and the number of animals used in each group are shown in panel A. Data are presented as means \pm SEM (error bar above). The letter "a" indicates a statistically significant difference (one-way ANOVA followed by Student–Newman–Keul's test; $p < 0.05$) when the different groups are compared to the group of animals receiving saline + AngII at each measurement period. The letter "b" indicates a statistically significant difference when the different groups are compared to rats receiving 20 nmol of Nor-BNI + AngII at each measurement period. The letter "c" indicates a statistically significant difference when the different groups are compared to the group of animals receiving saline + saline at each measurement period. Each bar in the graph was obtained from a naïve group of animals. panel C shows the percentage of total fluid consumed as water and saline at the end of the experimental session (120 min) in the same groups shown in panels A and B. * indicates a statistically significant difference (one-way ANOVA followed by Student–Newman–Keul's test; $p < 0.05$) when the percentage of water intake is compared with the percentage of hypertonic saline intake in each group.

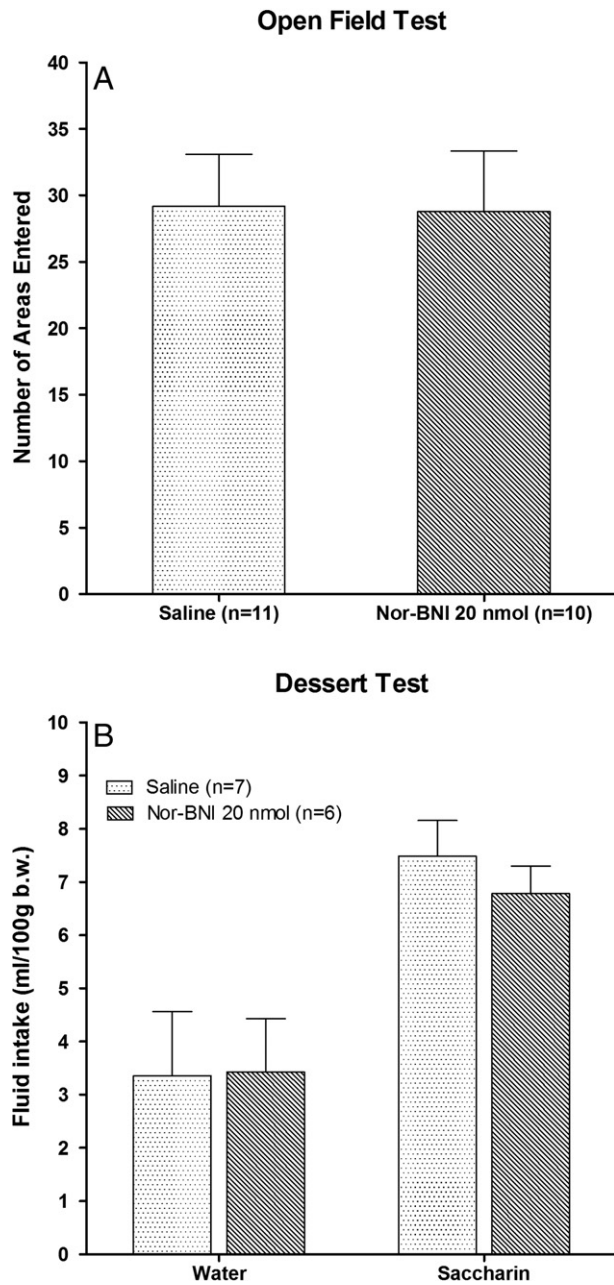


Fig. 4. Panel A shows the number of areas entered over a 15 min periods in the “open field” test carried out in rats receiving lateral ventricle injections of Nor-BNI (20 nmol) or isotonic saline solution (controls). The treatment received by each group and the number of animals used is indicated in the graph. Data are expressed as means + SEM (error bar above). There was no statistically significant difference (*t*-test; $p < 0.05$) between the group of animals receiving Nor-BNI and the saline-treated animals. Panel B shows cumulative saccharin and water intake (ml/100 g body weight) over a 2-h period in the test cage in rats receiving lateral ventricle injections of Nor-BNI (20 nmol) or isotonic saline solution (controls). The treatment received by each group and the number of animals used is indicated in the graph. Data are expressed as means + SEM (error bar above). There was no statistically significant difference (*t*-test; $p < 0.05$) when the group of animals receiving Nor-BNI was compared to controls.

is unaffected by the pharmacological blockade of central κ -opioid receptors; 5) the open field test showed that central blockade of κ -opioid receptors did not reduce locomotor activity; 6) the blockade of central κ -opioid receptors failed to modify blood pressure in animals submitted to sodium-depletion.

One of the procedures used here to induce salt intake was sodium-depletion, which was achieved by subcutaneous administration of the diuretic, furosemide. This is a classic model for inducing salt appetite and during the experimental sessions the animals had access to both

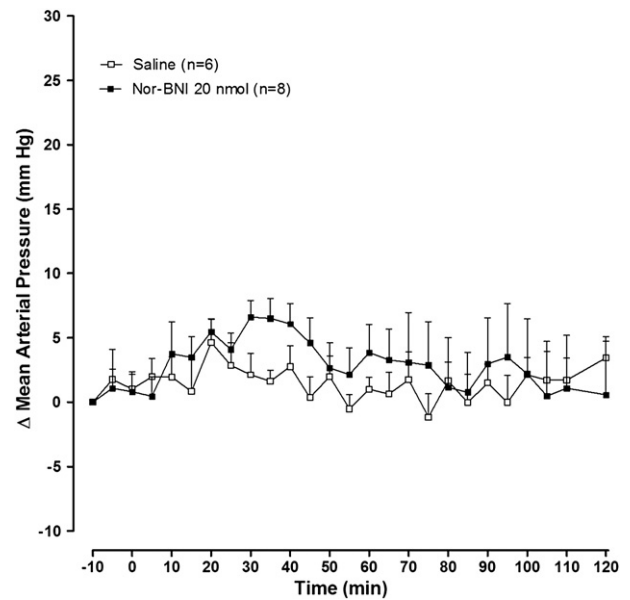


Fig. 5. Changes in mean blood pressure in sodium-depleted rats after injections of Nor-BNI (■ 20 nmol; $n = 8$) or saline solution (□; $n = 6$), into the lateral ventricle. Blood pressure recording between -10 and 0 min corresponds to a pre-drug baseline evaluation. The injections were given at 0 min and the bottles containing water and saline solution (1.5%) were immediately accessible. Data are presented as means + SEM (error bar above). There was no statistically significant difference between the groups (two-way ANOVA followed by Bonferroni test; $p < 0.05$).

distilled water and saline solution in a concentration that is usually aversive to rats [31,32]. Furosemide treatment depletes the animal of both sodium and water. With the experimental paradigm used here, water intake is permitted (overnight) prior to allowing access to saline solution. During the experiments, only salt intake was recorded, since water deficit was largely restored before access to saline was provided. In the two-bottle choice test between water and saline solution, as expected water intake was found to be very low compared to salt intake, which demonstrated a clear preference for salt. When sodium-depleted animals are treated with central injection of Nor-BNI at the doses of 10 (intermediate dose) and 20 nmol (highest dose), salt preference is inhibited. Although, there is a decrease in the total fluid intake, this is mainly due to the decrease in salt intake and not to a disproportional inhibition in salt and water intake.

Different brain neurotransmitters, including endogenous opioids, may mediate the palatability as well as the preference for specific macronutrients and fluid solutions [33–39]. The majority of studies have shown the involvement of central opioid peptides in regulating preference for sweet-tasting fluids [40,41]. However, some studies have also shown the participation of endogenous opioid peptides in salt preference. The blockade of opioid receptors with naloxone reduces the intake of hypertonic, hypotonic and isotonic saline solutions in water-deprived rats [16–19]. On the other hand, central stimulation of κ -, μ - and δ -opioid receptors increases saline intake in non-deprived rats [18,19,21]. In addition, in rats submitted to repeated sodium depletion, systemic injections of morphine increase hypertonic saline intake [22]. It has been shown that the quantity of saline solution ingested and the palatability of salty solutions are altered by repeated sodium depletion [23,24].

In the present study, central injection of Nor-BNI decreased the salt intake induced by sodium-depletion in a dose-dependent way. In contrast, another research group reported that central injections of Nor-BNI in fluid-deprived (24 h) animals failed to change either hypotonic or hypertonic saline intake [20]. In that study, the investigators recorded the amount of fluid intake only 3 h after a central injection of Nor-BNI, while in the present study salt and water intake

were measured sooner after central injections of Nor-BNI. Indeed, 15 min after the fluid bottles were reintroduced into the cages the amount of hypertonic saline intake was significantly lower in the animals treated with the highest dose of Nor-BNI (20 nmol) compared to saline-treated, sodium-depleted control animals. Although there was a slight increase in salt intake at the end of the experimental session (2 h) in Nor-BNI-treated animals, it never reached the amount of salt drunk by the control group. Another essential difference between the previous data and the present findings concerns the experimental protocol used to induce salt appetite, fluid deprivation and sodium-depletion respectively. These conditions may trigger diverse mechanisms that culminate with salt intake, which may explain the inhibition in salt intake in sodium-depleted animals, but not in fluid-deprived rats after κ -opioid receptors blockade. These data suggest that central κ -opioid receptor may modulate salt appetite in sodium-depleted rats.

The selectivity of Nor-BNI for κ -opioid receptors has been shown both *in vitro* and *in vivo* [42–44]. Nor-BNI is able to block the effect of the κ -opioid receptor agonist, ICI_{199,441} *in vivo* and *in vitro* [45,46]. Also, the effect of another κ -opioid receptor agonist U69,593 on neuronal activity is blocked by Nor-BNI with an apparent equilibrium dissociation constant (K_i) of 0.26 nM, as determined by the Schild analysis in the electrophysiological assay. Furthermore, in a binding study, Nor-BNI presented a K_i of 0.24 nM, as measured by radioligand binding displacement [42]. The κ -opioid receptor agonist, ICI_{199,441}, has been shown to have a high affinity and selectivity for the κ -opioid receptors and is 146-fold more active than U-50488 *in vitro* in the mouse vas deferens model. Furthermore, it was found to exert potent naloxone-reversible analgesic effects (ED_{50} = 0.004 mg/kg sc) in an abdominal constriction model [47,48]. In the present study, central administration of the κ -opioid receptor agonist, ICI_{199,441}, was unable to alter salt intake in sodium-depleted rats. However, since sodium-depleted rats drink a large amount of hypertonic saline, it is unlikely that pharmacological stimulation of κ -opioid receptors would further increase the volume of hypertonic saline intake. In contrast, in sodium-depleted rats pretreated with Nor-BNI, a competitive antagonist of κ -opioid receptors, and receiving central administration of ICI_{199,441}, the inhibitory effect of the central κ -opioid receptors blockade was reverted. These physiological data from the present study suggest that the ICI_{199,441} may have displaced the binding of Nor-BNI to the κ -opioid receptors. However further pharmacological and/or biochemical studies need to be performed to confirm this hypothesis.

In the present study, the effect of κ -opioid receptors on ingestive behavior seems to be specific for sodium appetite since the water intake induced by AngII remained unchanged by Nor-BNI pretreatment, while salt intake was inhibited in a dose-dependent way. Analyzing the percentage of water and salt intake relative to the total amount of fluid intake, it is clear that central administration of Nor-BNI at the doses of 10 and 20 nmol inhibits the preference for salt induced by central AngII stimulation. These data confirm the functional effect of κ -opioid receptors specifically on the control of salt appetite.

The interaction between opiate and angiotensinergic systems has already been shown, both at central and peripheral levels. At peripheral level, naloxone has been shown to attenuate the pressor response of an intravenous infusion of AngII [49]. Furthermore, systemic naltrexone inhibits water intake induced by subcutaneous AngII or hypertonic saline [50]. At central level, naloxone was found to inhibit water intake induced by AngII [51,52]. Moreover, the κ -opioid receptor antagonist, MR2266, injected intracerebroventricularly, prevents the pressor effect and the alteration of baroreceptor sensitivity induced by Ang II [53]. Confirming this functional interaction between the opiate and angiotensinergic systems, some morphological data have shown a reciprocal synaptic relationship between enkephalinergic and AngII neurons. In the area postrema, presynaptic enkephalin axon terminals were found to make synapses on the Ang perikarya and dendrites. On the other hand, AngII axon

terminals also make synapses on the enkephalin perikarya and dendrites, although this is less common than the aforementioned relationship between these two neuronal pathways [54]. Therefore, it is reasonable to affirm that AngII modulates endogenous opioid peptide release leading to increased sodium appetite, as suggested by the present data.

Understanding the role of opioid peptides in brain function is a rather complex matter. Endogenous opioids operate at many sites in the brain through four opioid families, enkephalins, dynorphins, endorphins and nociceptin/orphanin, interacting with different types of receptors distributed widely throughout the brain [1–7]. It is noteworthy that in areas of the brain related to the control of water and salt intake such as the septal and preoptic areas, paraventricular nuclei, lateral hypothalamus, periventricular nuclei and amygdala the density of μ , κ and δ receptors is moderate to high [2–4,55]. There is evidence that both dopaminergic and opiate systems in the nucleus accumbens and ventral tegmental areas are involved in appetitive behaviors and are associated with motivated behavior and drug-abuse [8,41,56,57].

In the present study, Nor-BNI had no effect on saccharin intake in the “dessert test”. This is in agreement with data published in the literature showing that intake of a palatable sucrose solution (10%) is reduced by injections of a μ -opioid receptor antagonist but not a κ -opioid receptor antagonist into the nucleus accumbens and ventral tegmental area [39,58,59]. Opioid peptides acting in the nucleus accumbens and ventral tegmental area have been shown to modulate the hedonic/reward processing of ingestive behavior based on the palatability of substances, while opioid peptides acting in the basolateral amygdala are involved in the motivational and learning process of ingestive behavior [60]. With respect to salt appetite, delta-opioid receptors at the nucleus accumbens and ventral tegmental area have been shown to participate in the control of salt intake [61]. In the present study, the blockade of κ -opioid receptors appears not to alter the palatability of the sodium solution (1.5%), and the inhibitory effect of Nor-BNI on salt intake may involve a reward-independent mechanism. However, further studies will have to be performed to clarify the role of different opioid receptors in the brain areas controlling salt appetite.

It has been shown that locomotor activity may be affected by opioid peptides. In general, μ - and δ -opioid receptors are associated with hyperactivity, while activation of κ -opioid receptors decreases locomotion [62]. In order to test the hypothesis that the decrease in salt intake caused by the blockade of κ -opioid receptors was not due to a general inhibition of locomotor activity, the open field test was performed in the present study. Results showed that locomotor activity was similar in sodium-depleted animals receiving intracerebroventricular injections of Nor-BNI and in the sodium-depleted control animals. Therefore, the inhibition of salt appetite induced by the blockade of κ -opioid receptors is specific for this behavior and not a consequence of any locomotor impairment.

Endogenous opioids may also modulate cardiovascular responses, although published data are not consistent, with both hypotension and hypertension having been described [1,9,62]. In general, μ - and δ -opioid receptors reduce blood pressure and heart rate [61]. Concerning κ -opioid receptors, a hypertensive effect was observed following blockade of the κ -opioid receptors in anesthetized rats, whereas in unanesthetized animals no change in blood pressure was found [53,63,64]. In the present study, intracerebroventricular injections of Nor-BNI had no effect on blood pressure in sodium-depleted rats; hence the antinatriuretic effect induced by κ -opioid receptor blockade is not due to a hypertensive response.

The data obtained in the present study may have clinical relevance since both agonists and antagonist of κ -opioid receptors have been used in different clinical intervention. It has been shown that κ -opioid receptors are implicated in the control of food intake in animals models of bulimia and binge-like eating [35–37] and pain mechanism and the

mediation of central and peripheral antinociception [65–68]. Furthermore, pharmacological manipulation of central κ -opioid receptors has been used in current clinical practice to detoxify patients addicted to opioids and maintain abstinence from illicit drugs [69–73]. In addition, κ -opioid receptor antagonists may prove useful for the treatment of depression, while κ -opioid receptor agonists may be useful for the treatment of bipolar disorder or mania [74].

In conclusion, these data show that the blockade of central κ -opioid receptors inhibits salt intake in two different models: sodium depletion and brain angiotensinergic stimulation. The decrease in salt intake does not appear to be due to a general inhibition of locomotor activity or to any change in palatability. The present study builds on and extends previous findings by Bodnar group [20] by indicating that particular physiological conditions, sodium-depletion and central angiotensinergic stimulation, are sensitive to κ -opioid receptor, whereas water deprivation is not.

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